Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at http://about.jstor.org/participate-jstor/individuals/early-journal-content.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

ON SPECIFIC ERYTHROPRECIPITINS (HEMOGLOBIN PRECIPITINS?)

LUDVIG HEKTOEN AND KAMIL SCHULHOF

From the John McCormick Institute for Infectious Diseases, Chicago

In 1901 Leblanc reported that beef hemoglobin acted as a specific precipitinogen, and this discovery was confirmed by Demees as well as by Ide, in whose laboratory the work was done.[1] Klein [2] found that injection of rabbits with watery extracts of red corpuscles gave rise to specific precipitins and Leers [3] confirmed while others either denied or doubted that specific erythroprecipitins could be produced.[3] Consequently, it seemed advisable to make new experiments, and we first studied precipitin formation in response to extracts of various corpuscles (table 1) on the same general plan as Klein and Leers.

The extracts are made by suspending the carefully washed corpuscles of a definite quantity of blood in many times that quantity of sterile distilled water, then adding as much again of 1.8% salt solution and centrifugating thoroughly. In this way clear solutions with 0.9% of sodium chloride are obtained; to insure sterility they may be passed through Berkefeld filters, which also removes the stromata and perhaps limits the number of antigens in the filtrates. In most cases the solutions we use are made so that 50 c c contain the extract of the corpuscles in one c c of blood. Five or 6 injections are given rabbits intravenously every 3 days, beginning with about 2 or 4 c c and increasing by about 2 c c each time. As usual, the precipitin content of the serum seems to reach the highest point 7-8 days after the last injection. The tests are made with progressive dilutions of erythrocytic extracts or serum in small tubes, a small quantity of precipitating serum being introduced at the bottom of each tube so as to give a precise plane of contact between the fluids. The results are read after one hour at room temperature, the

Received for publication March 1, 1922.

[1] Leblanc, A.: Contribution a l'étude de l'immunité acquise, La Cellule, 1901, 18, p. 335. Ide: Hémolyse et antihémoglobine, ibid., 1902, 20, p. 261. Demees, O.: Hémolyse et antihémoglobine, ibid., 1907, 24, p. 423. This excellent work has been overlooked to a large extent. Even Bordet in his Traité de l'Immunité, 1920, fails to mention the results of his compatriots.

[2] Wien. klin. Wchnschr., 1905, 18, p. 1055; Centralbl. f. Bakteriol., I, O., 1908, 39, p. 303.

[3] Centralbl. f. Bakteriol., I. O., 1910, 54, p. 362. For review of literature, see Uhlenhuth, P., u. Steffenhagen, K.: Die biol. Eiweiss-Differenzierung mittels der Präzipitat., Handb. d. path. Mikroorganismen, 1913, 3, p. 259; also Schmidt and Bennett, J. Infect. Dis., 1919, 25, p. 207.

titer or strength of a given antiserum being the highest dilution of the antigen in which distinct precipitate forms under the conditions outlined.

As a rule, the serum of rabbits injected as described with erythrocytic extracts contains small quantities only of precipitins for the corresponding serum proteins, sometimes hardly any at all. Such serum may contain also agglutinin, lysin and opsonin for the corpuscles that furnish the antigenic extract. The results illustrated in table 1 were obtained with antierythrocytic serums from which all or nearly all the precipitins for serum proteins had been removed by selective absorption. For the purpose of absorption, antiserum is mixed with an equal quantity of the

TABLE 1

PRECIPITINS FOR AQUEOUS EXTRACTS OF RED CORPUSCLES

Antigens: Extracts of Red Corpuscles and Corresponding Normal Serums	Precipitin Serums Produced by Injections of Extracts of Red Corpuscles as Follows					
	Beef	Dog	Horse	Human	Sheep	Swine
Extract of beef corpuscles	20,000	0	0	0	0	0
Beef serum	0	0	0	0	0	0
Extract of dog corpuscles	0	24,000	0	0	0	0
Dog serum	0	0	0	0	0	0
Extract of goat corpuscles	800	0	0	0	3,200	0
Goat serum	0	0	0	0	0	0
Extract of guinea-pig corpuscles	0	0	0	0	0	0
Guinea-pig serum	0	0	0	0	0	0
Extract of horse corpuscles	2,000	0	40,000	0	0	0
Horse serum	0	0	0	0	0	0
Extract of human corpuscles	800	0	0	5,000	0	0
Human serum	0	0	0	32	0	0
Extract of monkey (rhesus) corpuscles	800	0	0	3,200	..	0
Monkey serum	0	0	0	0	0	0
Extract of rabbit corpuscles	0	0	0	0	0	0
Rabbit serum	0	0	0	0	0	0
Extract of rat corpuscles	0	0	0	0	0	0
Rat serum	0	0	0	0	0	0
Extract of sheep corpuscles	800	0	0	0	6,400	0
Sheep serum	0	0	0	0	16	0
Extract of swine corpuscles	0	0	0	0	0	3,200
Swine serum	0	0	0	0	0	0

corresponding serum in a dilution of 1:200; this mixture is left at room temperature for one hour or so, placed in the icebox overnight and then centrifugated thoroughly. It should be noted that on account of this treatment the precipitin serums in table 1 are all diluted one-half. As stated, the figures in the table give the highest dilutions of extract and serums in which definite precipitiate formed; this is true also of table 2; in all cases save serums the figures give really the dilutions of the whole blood because in each case the unit is the extract or hemoglobin from the corpuscles in one volume of blood. The results speak for themselves. The erythroprecipitins produced with extract of dog, horse and swine corpuscles are strictly specific, being apparently species specific

as well as cell specific; in the case of the antiserum for extract of human corpuscles a small amount of precipitin for serum persists after absorption with human serum as described, and the action of the main precipitin is strongly marked on extract of monkey corpuscles; the sheep erythroprecipitin acts almost as well on extract of goat corpuscles as on extract of sheep corpuscles, and a small amount of precipitin for sheep serum persists after absorption; the precipitins produced in response to injections of beef corpuscles act on extract of goat, horse, human, monkey, and sheep corpuscles as well as on the extract of beef corpuscles and, as one might expect, in much higher dilutions of this last extract than of any of the others. In this case the precipitins are apparently cell specific but not strictly species specific, and the corpuscles of the mammals in question seem in some degree identical. We may return to the problem of the identity of antigens in the crude aqueous extracts of these mammalian corpuscles later. We would emphasize now that evidently red corpuscles contain antigenic elements that ordinarily do not occur to any marked extent in the corresponding serum, and the question arises: What may the nature of these erythrocytic constituents be?

First the effect of passing illuminating gas and H_2S through extracts of red corpuscles was tested, and it was found that this treatment in no way interfered with the precipitation of specific antiserum. Conversion of hemoglobin into methemoglobin was also without effect.

The next step was to study the effects of splitting the hemoglobin in the extract into hematin and globin. It was quickly shown that treating erythrocytic extracts with hydrochloric acid destroyed the elements concerned in the precipitin reaction, except with highly dilute solutions of the acid, and even then there was considerable loss. With acetic acid, however, followed by ammonia, splitting of the hemoglobin with preservation of a definite specific element was accomplished. Using a 1:50 extract (on the basis of whole blood) of human corpuscles, we mixed 30 c c of extract with 20 c c of a 1/10 N acetic acid; to small quantities of this mixture increasing quantities of 1/10 N ammonia were added and enough NaCl to make a 0.9% solution. The slightly acid, clear mixtures of this kind gave precipitation with the antiserum in as high dilutions as the untreated extract and no reaction with normal rabbit serum; making the reaction alkalin and removing the voluminous precipitate of the globulin together with a part of hematin by centrifuga-

tion, still left the specific precipitinogenic elements in the solution without perceptible diminution. Near the point of neutralization there was, however, some loss of precipitinogens.

Similar results have been obtained with extracts of horse, beef, sheep and swine corpuscles. In no case did globin itself dissolved in the smallest possible amount of 1/10 N acetic acid followed by partial neutralization, or in neutral solution obtained by dialyzing, give any specific precipitate with antiserum. As yet we have not succeeded in separating the antigen absolutely from all traces of hematin as the treatment necessary with ether alcohol destroyes the antigen, but the efforts are being continued. Of course, hematin itself hardly can be the antigen because it is a comparatively simple compound, probably identical in every species, and Schmidt and Bennett [4] state that Gay on direct tests obtained no evidence of hematin being antigenic. Furthermore, in our experiments the quantity of antigen was not reduced as part of the hematin was removed with the globin precipitate.

Treating antiserum with equal quantities of the corresponding original extract or of solution, from which the globin had been removed in the manner described, diluted in either case to correspond to 1:200 of the whole blood, resulted in each instance in the complete removal of all the precipitins in the antiserum, thus indicating that the globin-free solution contained practically all the antigenic elements in the original extract.

Injecting rabbits with repeated amounts of the globin-free extracts of corpuscles resulted in the production of antiserums that seem to be fully as specific in reaction as the antiserums produced by injecting the original extracts, being limited in their action to globin-free derivatives of extracts and the extracts themselves.

We next turned our attention directly to the question of the relation of hemoglobin itself to the antigenic properties of extracts of red corpuscles. Horse hemoglobin recrystallized 4 times by the ammonium sulphate method reacted with the antiserum in about as high a dilution as the original extract. In this treatment most of the hemoglobin was turned into methemoglobin. The Marshall-Welker [5] aluminum cream method is believed to remove all proteins other than hemoglobin from solution, but even after this treatment, repeated 4 times, horse, beef, sheep, swine and human hemoglobin reacted with their respective antiserums just as before; and removal of the globin from hemoglobin,

[4] Jour. Infect. Dis., 1919, 25, p. 207.
[5] Jour. Am. Chem. Soc., 1913, 35, p. 820.

purified with aluminum cream, by means of acetic acid and ammonia still left the precipitinogenic elements in the globin-free solutions. Treatment of globin-free solutions, diluted to correspond to 1/100 of blood, with aluminum cream resulted, however, in the complete removal of the precipitinogens, which appear to be protected against the action of this cream only when hemoglobin as such is present.

TABLE 2

SPECIFIC PRECIPITINOGENIC EFFECTS OF HEMOGLOBIN AND GLOBIN-FREE SOLUTIONS

Antigens	Antiserums Produced by Injecting Rabbits								
	Beef		Horse		Human		Sheep		Swine
	Hemoglobin	Globin-free Solution	Hemoglobin	Globin-free Solution	Hemoglobin	Globin-free Solution	Hemoglobin	Globin-free Solution	Hemoglobin
Beef:									
Hemoglobin	50000	12800	0	0	0	0	0	0	0
Globin-free solution	12800	12800	0	0	0	0	0	0	0
Serum	10	40	0	0	0	0	5	10	0
Horse:									
Hemoglobin	0	0	5000	4000	0	0	0	0	0
Globin-free solution	0	0	4000	2000	0	0	0	0	0
Serum	0	0	0	0	0	0	0	0	0
Human:									
Hemoglobin	0	0	0	0	6400	6400	0	0	0
Globin-free solution	0	0	0	0	3200	3200	0	0	0
Serum	0	0	0	0	320	320	0	0	0
Monkey:									
Hemoglobin	0	0	0	0	3200	800	0	0	0
Sheep:									
Hemoglobin	0	0	0	0	0	0	50000	25000	0
Globin-free solution	0	0	0	0	0	0	25000	25000	0
Serum	0	0	0	0	0	0	0	0	0
Goat:									
Hemoglobin	0	0	0	0	0	0	25000	12800	0
Swine:									
Hemoglobin	0	0	0	0	0	0	0	0	50000
Globin-free solution	0	0	0	0	0	0	0	0	12800
Serum	0	0	0	0	0	0	0	0	0

As 100% on Sahli's scale corresponds to 17.3 grams hemoglobin in 100 c c, the actual amount of hemoglobin in different solutions may be computed by means of the hemoglobinometer. A solution on the basis of blood 1 to 50 corresponds approximately to a solution of one part of hemoglobin in 300. Consequently, if the figures for hemoglobin in this table are multiplied by 6 the products will indicate the approximate dilution of pure hemoglobin in each case.

By injecting rabbits with the purified hemoglobins of the species mentioned and with the globin-free solutions thereof, we obtained strictly specific precipitin serums whose action is limited to the hemoglobin and globin-free solutions used as antigens in each case as shown in table 2. In case of the human antigens the antiserum formed precipitate with a low dilution of the corresponding serum due possibly to the hemoglobin in the serum which gave a positive benzidin reaction. These antiserums do not contain any agglutinin, lysin or opsonin for the

corpuscles that furnish the antigenic hemoglobin, a fact of some interest in connection with the question whether various antibody effects depend on one or several distinct substances.

DISCUSSION

Hemoglobin has been the object of many investigations, but only few deal with the question whether it is responsible for the antigenic properties of extracts of red corpuscles. Demees [1] purified hemoglobin by the ammonium sulphate method and tried to determine its relation to the production of hemolysin; he obtained precipitins which he attributed to the antigenic action of hemoglobin, but no lysin, and he noted especially that a yellow precipitate developed under certain conditions. Our results appear to confirm Demees. In the more recent investigations of the antigenic properties of hemoglobin and its protein constituent, globin, diametrically opposite results have been obtained. In the course of our work certain reasons for this divergence have appeared.

First, we find that hydrochloric acid, except in very weak concentration, destroys the antigenic property of the aqueous extract of erythrocytes. Gay and Robertson [6] concluded from complement fixation and anaphylaxis tests that globin is not antigenic, but that it may be rendered so in a compound with casein. Schmidt [7] found that a globin-albumose compound is not antigenic. Now, as the preparations of globin used in these investigations were obtained by strong hydrochloric acid (20 c c of concentrated acid to 1 liter of solution), it is evident that the results do not exclude the possibility of unchanged globin being antigenic. On the other hand, Browning and Wilson,[8] working with a much weaker solution of hydrochloric acid, came to the conclusion that antibodies against globin may be obtained, which, however, did not react with hemoglobin. We find that the antigen remains in solution after removing practically all the globin, but near the neutralization point the globin precipitate usually carries down a great deal of the antigen, and this may account for the results obtained by Browning and Wilson, who used partly an acid solution, partly an alkaline emulsion, the acid solution containing besides the globin the unknown antigen in solution and the alkaline emulsion a varying part of it partly dissolved and perhaps partly adsorbed to globin.

Browning and Wilson used complement fixation and not the precipitin method, because the globin precipitated all serums, but it does so only in stronger solutions, and all chances of error may be eliminated

[6] Jour. Exper. Med., 1913, 17, p. 535.
[7] Univ. of Calif. Public. in Pathology, 1916, 2, p. 157.
[8] Jour. Path. Bacteriol., 1909, 14, p. 174.

by controls with normal rabbit serum. Besides the hematin present seems to protect the serum. We believe that the precipitin method, generally speaking, gives more satisfactory results than the other methods, besides being much simpler.

Schmidt and Bennett [4] prepared hemoglobin by repeated crystallization. The two methods they used, namely, 15% and 25% alcohol, do not permit the conclusion that hemoglobin is not antigenic, because alcohol we find is injurious to the antigen, especially in the presence of salts. These authors apparently were convinced that the negative results of their third method, namely ammonium sulphate, would not be decisive as the hemoglobin now was changed into methemoglobin, but we find that a solution in which hemoglobin has been changed into methemoglobin retains the antigenic properties of the original extract. Even after four recrystallizations followed by carbon dioxid treatment (Schmidt and Bennett's third method), horse hemoglobin (methemoglobin) reacted with antiserum as strongly as originally. It is possible that they might have obtained positive results had they injected more than 2 rabbits to produce antibodies. In any case, it would seem to be a good plan to begin by testing the different solutions with an antiserum previously prepared with the original extract.

Of other contributions to the study of the antigenic action of hemoglobin may be mentioned the work by Bradley and Sansum,[9] in which they obtained positive specific results from anaphylactic experiments with hemoglobin, and the recent work by Higashi [10] on the antigenic action of hemoglobin in which he appears to show that the precipitinogenic properties are retained by hemoglobin when eliminated in the urine. He holds that Klein's erythrocytic precipitin is a hemoglobin precipitin. Azuma [11] is said to have shown that hemoglobin precipitin is specific and applicable to medicolegal tests, a question that we may take up later. On account of its precipitin strength and the sharp limitation in action, antihemoglobin serum may prove of value as a test for blood in feces.

Our results appear to indicate that hemoglobin may be a specific antigen and thus they may help to throw light on its constitution. In extension of the work, the isolation of the unknown antigen and its relation to native hemoglobin are problems that demand special attention. At present we believe the following conclusions are justified:

[9] J. Biol. Chem., 1914, 18, p. 497.
[10] Jap. Med. World, 1922, 2, p. 52.
[11] Ibid., p. 85.

SUMMARY

Aqueous extracts of red corpuscles give rise in rabbits to precipitins the action of which appears to be limited to erythrocytic constituents, in some cases of the species furnishing the corpuscles only, in other cases extending also to such constituents of related species.

While other antigens may be present in crude aqueous extracts of corpuscles, the main precipitinogen seems to be hemoglobin, which is shown to be a species specific precipitinogen in confirmation of the early work of Ide and his pupils.

Conversion of the hemoglobin in extracts of red corpuscles into carboxyhemoglobin, sulphydrohemoglobin or methemoglobin does not affect the specific serum precipitation of the hemoglobin.

On splitting hemoglobin into hematin and globin by means of acetic acid, the precipitinogenic elements remain in the solution after removal of the globin, which does not appear to be responsible for the antigenic properties of the hemoglobin, the globin-free solution, however, being antigenic not only in tests with antiserum but also on injection in rabbits.

While the precipitinogens in extracts of red corpuscles and in hemoglobin may exist independently of hemoglobin after treatment with acids, they ordinarily are attached closely to the hemoglobin molecule, not being removed or diminished in proportion to the amount of hemoglobin by repeated crystallization or by treatment with aluminum cream, the antigen being apparently either closely adsorbed to the hemoglobin molecule or forming a part of it which can be split off by acids.